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Rapid and Specific Identification of *Yersinia pestis* by Using a Nested Polymerase Chain Reaction Procedure

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We developed a 4-h nested polymerase chain reaction assay that detected a region of the plasminogen activator gene of *Yersinia pestis* in 100% of 43 *Y. pestis* strains isolated from humans, rats, and fleas yet was unreactive with the closely related species *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*.

Rapid detection of *Yersinia pestis* with DNA probes is possible if suitable gene sequences, such as those encoding species-specific virulence factors, can be targeted (5). In this report, we describe a polymerase chain reaction (PCR)-based procedure that detects *Y. pestis*-specific plasminogen activator gene sequences present in bacterial isolates from humans, nonhuman mammals, and fleas.

All bacterial isolates except *Escherichia coli* HB101 were obtained as lyophilized cultures from the archived collection of the U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, Md. These lyophilized cultures had been maintained in ultralow-temperature storage for periods of up to 46 years. The 43 *Y. pestis* isolates used in this study had been recovered from humans, nonhuman mammals, and fleas from diverse geographic locations throughout the world (Table 1). Other lyophilized bacterial isolates used included one strain each of *Yersinia enterocolitica* (WR E261) and *Yersinia pseudotuberculosis* (ATCC 6003). Lyophilized specimens were rehydrated in 0.2 to 5.0 ml of 0.9% NaCl. Samples of these rehydrated bacteria were used directly in PCR tests without prior culturing.

Genomic DNA for PCR tests was prepared from human lymphocytes, rat liver tissue, and two species of fleas (*Diamanus montanus* and *Hoplopsyllus anomalus*) documented as major vectors of plague in the southwestern United States (2) by first grinding the tissues in LN_2 with a mortar and pestle. The tissue powder was immediately transferred into approximately 10 volumes of extraction buffer (10 mM Tris-Cl [pH 8.0], 0.1 M EDTA [pH 8.0], 20 μg of pancreatic RNase per ml, 0.5% sodium dodecyl sulfate), mixed, and incubated at 37°C for 1 h. Proteinase K was then added to a final concentration of 100 μg/ml, and the mixture was incubated at 50°C for 3 h. The solution was extracted with phenol and was ethanol precipitated.

Two nested sets of optimized oligonucleotide primer pairs for PCR were selected from regions of the plasminogen activator gene of *Y. pestis* (6). The outer primers, which amplified a DNA fragment of 928 bp, were designated YP1 (AAGTTCTATTGTGGCAACC) and YP2 (GAAGCGATAT TGCAGACC). The inner nested primers, which amplified a DNA fragment of 458 bp, were designated YP1a (CTGACA GCTTTACAGTTGCAGC) and YP2a (CACTCCTTTCGGG AAGTTTCCG). Another primer pair was similarly selected to amplify a region of the yersinial outer membrane protein gene, *yopl*, of *Y. pseudotuberculosis* and *Y. enterocolitica* (3).

Rehydrated bacterial suspensions, genomic DNA, and turbid suspensions of *E. coli* HB101 were the sources of template DNA in PCR tests. Samples were denatured at 94°C for 1 min, primers were annealed at 58°C for 1 min, and new DNA was extended at 72°C for 1 min. After amplification, a 1:10 dilution of the reaction mixture was used as the template for a second round of PCR with the nested set of primers. All test and control samples that failed to yield PCR fragments on the first round of amplification were retested by a modified PCR protocol that included a preliminary denaturing step of 5 min at 94°C ("hot start") before the cycling described above. The two control *Yersinia* isolates were also subjected to PCR with primers specific for the *yopl* gene according to the same protocol used with the *Y. pestis* primers.

A 458-bp PCR fragment obtained by amplification of DNA from *Y. pestis* YEO614 with the plasminogen activator gene-specific primers YP1a and YP2a was separated from the reaction mixture by electrophoresis through a 0.7% agarose gel. The fragment was excised from the gel, electroeluted, ethanol precipitated, and used as the double-stranded template in overlapping sequence reactions with the same primer set to generate either forward (YP1a) or complementary (YP2a) DNA sequences.

Of the 43 different lyophilized *Y. pestis* isolates tested, 39 (91%) yielded the predicted 928-bp amplification product after a single round of PCR with the outer (YP1 and YP2) primer set. The intensity of these amplified fragment bands was quite variable. The results of 12 representative PCR amplifications are presented in Fig. 1A, lanes 7 to 18. All 43

TABLE 1. *Y. pestis* isolates used in this study

Geographic location	Isolates
New World	YEO149, -150, -225, -244, -386[a], -387, -471, and -474
Asia	YEO147, -154[a], -161, -165, -189, -215, -221, -233, -234, -238, -240, -307[a], -316[b], -336, -339, -524, -529, and EV76
Africa	YEO158, -160, -201, -237[a], -499, -570[b], -578, -579, -583[b], -587, -592, -605[a], -609[b], -614, and -616
Unknown	YEO145 and -947

[a] *Y. pestis* isolate from a nonhuman mammal.
[b] *Y. pestis* isolate from fleas.

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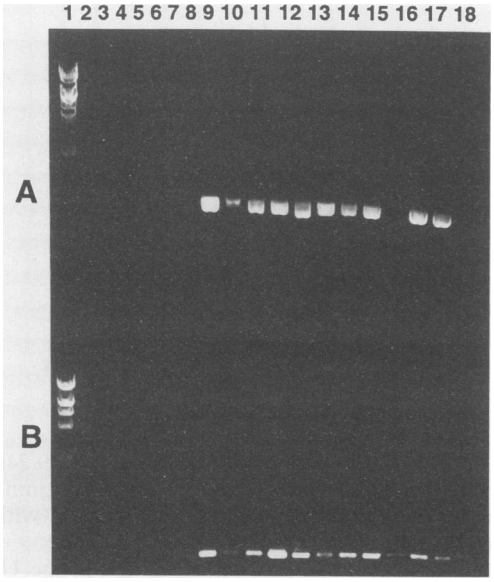

FIG. 1. Agarose gel analysis of PCR products with primers YP1 and YP2 only (A) and YP1 and YP2 followed by YP1a and YP2a (B). Lanes: 1, lambda-*Hin*dIII molecular weight markers; 2, human DNA; 3, rat DNA; 4, flea DNA; 5, *Y. enterocolitica*; 6, *Y. pseudotuberculosis*; 7 to 18, *Y. pestis* isolates. The faint, low-molecular-weight bands in lanes 9 and 12 represent mispriming.

Y. pestis isolates tested by the two-step nested PCR procedure produced the predicted 458-bp amplification product (data for 12 representative amplifications are shown in Fig. 1B, lanes 7 to 18). The nested PCR procedure required only 4 h to complete. Among these PCR-positive *Y. pestis* strains was one atypically urease-positive isolate. PCR amplification of the closely related specificity controls *Y. pseudotuberculosis* and *Y. enterocolitica*, as well as *E. coli*, with primers specific for plasminogen activator gene sequences in either one-step or nested two-step protocols (Fig. 1, lanes 5 and 6 in both panels) failed to produce any product. However, PCR amplification of these two control bacteria with primers specific for the *yopl* gene of *Y. pseudotuberculosis* and *Y. enterocolitica* produced the predicted amplification product (data not shown). Host and vector control DNAs, purified from human lymphocytes, rat liver tissue, and total flea homogenates, were also subjected to PCR in the presence of *Y. pestis* primers, with negative results (Fig. 1, lanes 2 to 4 in both panels). Preheating samples to 94°C prior to subjecting them to PCR did not alter negative results (data not shown).

The DNA sequence of the PCR product was 100% homologous with the published sequence at the corresponding position on the plasminogen activator gene of *Y. pestis*.

This study describes a technical method in which *Y. pestis*-specific gene targets were detected within 4 h in 100% of 43 lyophilized *Y. pestis* isolates tested. The two-step nested PCR procedure failed to yield detectable amplification fragments with any of the control DNAs, including samples containing either of the closely related species *Y. enterocolitica* and *Y. pseudotuberculosis*. The observation that the one atypically urease-positive *Y. pestis* isolate tested in our study was clearly identified by PCR has potential clinical relevance because *Y. pestis* is usually urease negative (1), and urease activity is a key test in the biochemical differentiation of *Y. pestis* from *Y. enterocolitica* and *Y. pseudotuberculosis* (4). Future evaluation of the method with samples of infected fleas or patient serum samples may demonstrate its utility as a new clinical or epidemiological tool.

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REFERENCES

1. **Brubaker, R. R.** 1979. Expression of virulence in yersiniae, p. 168–171. *In* D. Schlessinger (ed.), Microbiology—1979. American Society for Microbiology, Washington, D.C.
2. **Butler, T.** 1983. Plague and other *Yersinia* infections, p. 45. *In* W. B. Greenough and T. C. Merigan (ed.), Current topics in infectious disease. Plenum Press, New York.
3. **Emödy, L., J. Heesemann, H. Wolf-Watz, M. Skurnik, G. Kapperud, P. O'Toole, and T. Wadström.** 1989. Binding to collagen by *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*: evidence for *yopA*-mediated and chromosomally encoded mechanisms. J. Bacteriol. **171**:6674–6679.
4. **Krieg, L. (ed.).** 1984. Manual of systematic bacteriology. Williams and Wilkins, New York.
5. **Plano, G. V., S. S. Barve, and S. C. Straley.** 1991. LcrD, a membrane-bound regulator of the *Yersinia pestis* low-calcium response. J. Bacteriol. **173**:7293–7303.
6. **Sodeinde, O. A., and J. D. Goguen.** 1989. Nucleotide sequence of the plasminogen activator gene of *Yersinia pestis*: relationship to *ompT* of *Escherichia coli* and gene *E* of *Salmonella typhimurium*. Infect. Immun. **57**:1517–1523.